50

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/127, C12N 15/09	A1	(11) International Publication Number: WO 96/40067 (43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09954

(22) International Filing Date: 7 June 1996 (07.06.96)

(30) Priority Data:
08/485,866 7 June 1995 (07.06.95) US

(71) Applicant: ARONEX PHARMACEUTICALS, INC. [US/US]; 3400 Research Forest Drive, The Woodlands, TX 77381 (US).

(72) Inventors: WYSE, Joseph, W.; 175 E. Pathfinders Circle, The Woodlands, TX 77381 (US). WARNER, Charles, D.; 38 Lush Meadows Place, The Woodlands, TX 77381 (US).

(74) Agent: SAUNDERS, Thomas, M.; Lorusso & Loud, 440 Commercial Street, Boston, MA 02109 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CATIONIC LIPID ACID SALT OF 3 BETA [N-(N',N'-DIMETHYLAMINOETHANE)-CARBAMOYL] CHOLESTEROL

(57) Abstract

This invention discloses a novel cationic lipid acid salt of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol. This invention further discloses a transmembrane compatible body suitablefor transfection of animals and animal cells with nucleotides such as DNA, RNA, and synthetic nucleotides. Such transmembrane compatible bodies arise from hydratable non-liposomal halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol and DOPE. This invention yet further discloses a halogenated solvent-free aqueous solution, suitable for lyophilization into a preliposomal powder, wherein the solution comprises 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol wherein substantially all 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol is dissolved.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

CATIONIC LIPID ACID SALT OF 3 BETA [N-(N',N'-DIMETHYLAMINOETHANE)-CARBAMOYL] CHOLESTEROL

Field of the Invention

This invention discloses a novel cationic lipid acid salt of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol. This invention further discloses a transmembrane compatible body suitable for transfection of animals and animal cells with nucleotides such as DNA, RNA, and synthetic nucleotides. Such transmembrane compatible bodies arise from hydratable non-liposomal, halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol. This invention yet further discloses a halogenated solvent-free aqueous solution suitable for lyophilization into a preliposomal powder, wherein the prelyophilate solution comprises 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol and dioleoyl phosphatidylethanolamine (DOPE) wherein substantially all 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol and DOPE are dissolved.

Background of the Invention

Liposomal gene therapy (a concept which includes transfection or gene delivery) is a powerful mechanism for the delivery of DNA and RNA as well as synthetic congeners thereof. In particular instances, the nucleic acid so delivered is bioactive as antisense, missense, nonsense, as protein producers, on and off and rate regulatory switching for protein or peptide production. Such delivery is frequently for therapeutic or diagnostic purposes in or directed to humans and human cells, and more broadly to animals, animal cells, and the disease and/or metabolic states of animals and animal cells. In this context, the term "animals" is expansively understood to included the entire phyla.

The fields of liposome and gene research have dramatically changed since the introduction of cationic liposomes in 1987 (Felgner et al., Proc. Natl. Acad. Sci. U.S.A., 84:7413-7417 (1987)). Liposomal DNA delivery has been

noted in Brigham et al. Am. J. Respir. Cell. Mol. Biol., 1:95-100 (1989); Burger et al. Proc. Natl. Acad. Sci. U.S.A., 89:2145-2149 (1992); Felgner and Ringold, Nature, 337:387-388 (1989); and, Muller et al., DNA Cell Biol., 9:221-229 (1990). Malone et al., disclose mRNA delivery Proc. Natl. Acad. Sci. U.S.A., 86:6077-6081 (1989), as does Weiss et al., J. Virol., 63:5310-5318 (1989). Protein delivery is discussed in Debs et al., J. Biol. Chem, 265:10189-10192 (1990); Nair et al., J. Exp. Med. 175:609-612 (1992); and Walker, Proc. Natl. Acad. Sci. U.S.A., 89:7915-7919 (1992). Delivery of antisense oligomers is reported by Bennett et al., Mol. Pharmacol., 41:1023-1033 (1992); and Chiang et al., J. Biol. Chem., 266:18162-18171 (1991). Particular liposomal formulations are cited in Felgner et al., J. Biol. Chem., 269:2550-2561 (1994); Levintis and Silvius, Biochem. Biophys. Acta, 1023:124-132 (1990); and, Ito et al. Biochem. Intl.. 22:235-241 (1990).

U.S. Pat. 5,283,185 to Epand et al., discloses 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol in basic form for use in transfection. Basic 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol A) is not soluble in aqueous solutions. Previous reports of DC cholesterol misattribute a positive charge to the nitrogen of the dimethylamine, which is, in fact neutral. (Fig. 1)

Preparing a liposome suspension of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol from all previously available aqueous insoluble forms required preparation from a dried thin film. This process required solubilization of the basic 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol in chloroform. a halogenated solvent. For pharmaceutical applications, residues of halogenated solvents cannot be practically removed from a preparation after being introduced. The inability to provide a halogen-free preparation has hampered the development of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol for use in liposome-type transfecting therapy as a transfecting agent.

Sterile preparation of a liposomal suspension, in some instances (such as from a thin film preparation), requires a mechanical shearing to reduce the

liposomal size to less than 0.2μm for sterile filtration. This is accomplished by sonication or passing the liposomes through a french press device such as a Microfluidizer Model M11-S (Microfluidic International Corp., Newton, MA.). While considering liposomes prepared from 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol in basic form, U.S. Pat. 5,283,185, to Epand et al., do not disclose the lyophilization of such liposomes. Lyophilization of liposomes prepared from 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol in basic form requires the addition of cryoprotectants such as trehalose to maintain reconstitutability of liposomes.

Brief Description of the Drawings

Fig. 1 is a diagram of the chemical structure of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol ("DC-chol B").

Fig. 2 is a flow diagram of the synthesis of DC-chol B hydrochloride.

Fig. 3 is a time, temperature, pressure graph of particular lyophilization cycle.

Fig. 4 is a size and stability over time data presentation of TCB/DNA complexes after mixing.

Summary of the Invention

This invention comprises the acid salt of the lipid 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol ("DC-chol B"), with particular reference to hydrochloride (HCl) salt.

In another embodiment, this invention comprises a transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol, with particular reference to the cationic lyophilate and wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is an acid salt. This invention further includes such lyophilate wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol acid salt is the hydrogenchloride salt. Optionally such lyophilate includes a lipid selected from the group consisting of dioleoyl

phosphatidylethanolamine ("DOPE") or dioleoyl phosphatidylcholine (DOPC). In particular embodiments the DOPE constitutes from about 20 mol% to about 80 mol% of the total lipid of the lyophilate, or from about 30 mol% to about 50 mol%. In certain embodiments of the lyophilate the DC-chol constitutes from about 50 mol% to about 70 mol% of the total lipid of the lyophilate. The lyophilate optionally further includes a sugar-class compound, such as dextrose, sucrose, lactose, mannose, and xylose, and particularly mannitol.

In some embodiments this invention comprises a halogenated solvent-free transfecting transmembrane compatible body comprising a 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol, and optionally further comprising DNA*. In particular embodiments of halogenated solvent-free transfecting transmembrane compatible body, DOPE is further included. It is understood that the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is basic in some embodiments, and can also be an acid salt (such as the hydrogen chloride salt), or mixtures of the two, and optionally further comprising DNA*.

In still further embodiments, this invention comprises a halogenated solvent-free aqueous solution comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol wherein substantially all 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is dissolved, and further wherein the solution optionally comprises tBA, particularly wherein the ratio of water to tBA is from about 70:30 to about 0:100, or wherein the percentage of water to tBA is at least about 70%. Reference is also made to such solution wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is at least about 25% DC-chol B, or at least about 40%, or at least about 60%. Such solution optionally comprises a sugar-class compound, such as dextrose, sucrose, lactose, mannose, and xylose, and particularly mannitol.

This invention includes a halogenated solvent-free aqueous process for preparing a transmembrane compatible body hydratable non-liposomal lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol comprising the steps of

(a) solvating DC-chol in a halogenated solvent-free solvent;

(b) simultaneously or sequentially with step (a) solvating DOPE in said halogenated solvent-free solvent;

(c) lyophilizing said solutions of (a) and (b).

Reference is made to the use of DC-chol contemplates use of DC-chol A and B in a ratio of from about 0:100 to about 100:0, in this process, and particularly wherein said ratio is from about 20:80 to about 40:60. Further, this process contemplates DOPE as present from about 20 mol% to about 80 mol% relative total lipid in the lyophilate, particularly wherein DOPE is present from about 30 mol% to about 50 mol%. This process yet further optionally includes the step of dissolving a sugar-class compound such as dextrose, sucrose, lactose, mannose, and xylose, and particularly mannitol, and most particularly mannitol in the solution prior to lyophilization (simultaneously or sequentially with steps (a) and (b)). Such sugar-class compound is particularly useful in a ratio of from about 0.1% to about 7.5%, and particularly from about 0.5% to about 2%, and further, specific to mannitol, wherein the mannitol is present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5%, and specifically from about 0.5% to about 2%.

This invention entails a method of incorporating a sugar-class compound into a transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol comprising

(a) solvating DC-chol in an aqueous halogenated solvent-free solvent;

(b) simultaneously or sequentially with step (a) solvating DOPE in said halogenated solvent-free solvent;

(c) simultaneously or sequentially with steps (a) and (b) dissolving a sugar-class compound in said halogenated solvent-free solvent;

(d) lyophilizing said solutions of (a)-(c).

In this method the sugar-class compound is usefully selected from the group comprising dextrose, sucrose, lactose, mannose, and xylose, and particularly mannitol. Mannitol is also useful when present in the solution prior to

lyophilization in a ratio of from about 0.1% to about 7.5% or from about 0.5% to about 2%, and the sugar-class compound is particularly useful if present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5%, and specifically from about 0.5% to about 2%.

This invention includes a method of introducing bioactive DNA* into a cell comprising the step of exposing said cell to a halogenated solvent-free TCB/DNA*complex wherein said TCB comprises 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol and DOPE, and is absent halogenated solvent.

This invention yet further includes a transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising a lipid which is a cationic lipid acid salt, and optionally further comprising DOPE, and further optionally comprising a sugar-class compound such as dextrose, mannitol, sucrose, lactose, mannose, and xylose. In particular embodiments DOPE constitutes from about 20 mol% to about 80 mol% of the lyophilate as compared with total lipid.

Detailed Description of the Invention

For clarity, the following definitions are used herein:

A. 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol ("DC-chol B") shall mean a composition in the form of an acid salt as shown in Fig. 1 (there, as the hydrochloride salt, 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride) as compared with the basic form. DC-chol B has unique surfactant characteristics as compared to non-acid DC-chol. In addition, DC-chol hydrochloride has a strongly charged amine group. Fig 2. depicts a flow diagram for synthesis of DC-chol B hydrochloride.

B. Cationic lipids shall include cholesteryl-3β-carboxyamidoethylenetrimethylammonium iodine, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine, cholesteryl-3β-

oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysuccinate iodide, 2-[(2-trimethylammonio)ethylmethylamino]ethyl-cholesteryl-3β-oxysuccinateiodide, 3β[N-[(N',N'dimethylaminoethane)-carbamoyl]-cholesterol, and 3β[N-[(N',N'dimethylaminoethane)-carbamoyl]-cholesterol, and 3β[N-(polyethyleneimine)-carbamoyl]cholesterol.

C. Acid salts, as applied to cationic lipids shall be used expansively to mean the hydrogen chloride, and quaternary ammonium salts, and more generally the salts of organic and inorganic acids including but not limited to hydrofluoric, hydrobromic, sulfuric, p-toluenesulfonic and phosphoric acids.

Acid salts as applied to of DC-chol B shall be used expansively to mean the hydrogen chloride, and quaternary ammonium salts, and more generally the salts of organic and inorganic acids including but not limited to hydrofluoric, hydrobromic, sulfuric, p-toluenesulfonic and phosphoric acids. The following salts are particularly contemplated: 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol fluoride, 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol bromide; 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol iodide; 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol sulfate; 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol hydrogen sulfate; 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol p-toluenesulfonate; 3β[N-(N',N'-dimethylammonioethane)-carbamoyl]-cholesterol dihydrogenphosphate. Quarternary salts include 3β[N-(N',N'-Trimethylammonioethane)-carbamoyl]-cholesterol salt; 3β[N-(N',N'-Ethyldimethylammonioethane)-carbamoyl]-cholesterol salt; 3β[N-(N',N'-Butyldimethylammonioethane)-carbamoyl]-cholesterol salt; and 3β[N-(N',N'-dimethylpropylammonioethane)-carbamoyl]-cholesterol salt. In this context salts will encompass fluoride, chloride, bromide, iodide, hydrogen

sulfate, p-toluenesulfonate, dihydrogen phosphate, and tetrafluoroborate as anion.

D. Transmembrane compatible body ("TCB") shall mean a lipid bilayer body such as a liposome, as well as a micelle, an amorphous amphipathic lipid comprising particle, an aggregate, or an emulsion, any of which traverse a cell membrane under conditions not inconsistent with cell life. By "not inconsistent with cell life" it is understood that the mere conditions of traversal will not cause substantial cell death. This does not exclude the potential for the result of the TCB traversal to be directed to intentionally causing cell death, such as by elaboration of a toxic protein within a cell after such traversal. Nonlimiting examples of such traverse are phagocytosis, endocytosis, apoptosis, intercalation and fusion of the cell membrane with the TCB. In some embodiments, traversal is augmented by techniques such as electroporation or exposure of cell membranes to polyethylene glycol or other membrane solvents.

E. Hydratable shall mean that a powder, upon addition of suitable pharmaceutically acceptable aqueous medium such as water, isotonic saline, 5% dextrose solution, sodium lactate solution, Ringer's solution, Ringer's lactate solution or other aqueous physiologically compatible solutions or combination of solutions, and further including those containing nucleic acids, shall form transmembrane compatible bodies. The term "reconstitutable" is used in the literature when lyophilized liposomes are rehydrated into liposomes. In the present application, the terms "hydrate," "hydratable" and "rehydratable" are preferably employed to stress that the original material was non-liposomal before (as well as after) lyophilization, and until hydration, though in some instances, reconstitutable is used. In particular instances, hydration is accompanied by sonication, stirring or other forms of agitation and mixing.

F. Non-liposomal lyophilate shall mean that the solution lyophilized to form the claimed composition was not in liposomal form at the time of

lyophilization. That is, the solution was substantially liposome free before and after lyophilization, and until the point of hydration.

G. Halogenated solvent-free shall mean the absence of halogenated residues, that is less than about 1ppm. Halogenated residues typically arise from the use of halogenated solvents such as chloroform.

H. Sugar or sugar-class compound shall mean monosaccharides, disaccharides, and further be expansively defined to include sugar alcohols. Sugar-class compounds, include, without limitation, dextrose, mannitol, sucrose, lactose, mannose, and xylose. In the practice of this invention, sugars-class compounds useful as bulking agents for the preliposomal lypohilate. An additional advantage of using sugar-class compounds as bulking agents is that the reconstitution of the TCB may be simplified by reconstitution with sterile water, rehydrating the lipid and sugar thus resulting in a physiologically desirable osmolarity of the TCB suspension. The use of sugar-class compounds as cryoprotectants is not a relevant consideration in the present invention because the solutions from which the preliposomal lyophilates are made are specifically nonliposomal. Thus, there is no gross membrane structure to cryoprotect.

I. Transfecting (or Transfection) shall mean transport of DNA* from the environment external to a cell to the internal cellular environment, with particular reference to the cytoplasm and/or cell nucleus. Without being bound by any particular theory, it is understood that transported DNA* may be transported as encapsulated, within or adhering to one or more TCBs or entrained therewith. Particular transfecting instances deliver DNA* to a cell nucleus.

J. DNA* shall be expansively understood to include both DNA and RNA as well as synthetic congeners thereof. Such DNA* includes missense, and antisense, nonsense, as well as protein producing nucleotides on and off and rate regulatory nucleotides for control of protein and peptide production and on and off control of DNA* production. Particular, but

nonlimiting, reference is made to DNA, RNA, and oligonucleotides which are bioactive.

K. Bioactive as applied to DNA* indicates a relationship to a biological process. By way of nonlimiting examples, bioactive includes process which are initiated, accelerated, decelerated, inhibited, enhanced, maintained or otherwise modified, as well as affixation to biological sites resulting in blocking, marking or inactivation of biological processes. Bioactive shall further include diagnostic actions such as marking (*i.e.*, complement DNA probes).

L. Solvating in reference to lipids means dissolving such that there are substantially no liposomes upon said solvation.

M. Dissolving, in reference to sugar-class compounds, means that the sugar-class compound is brought into solution. In an non-aqueous solvent system, such as 100% tBA, additional water must be added to the solvent system to accomplish dissolving, and the addition of such amount of water is entailed in this definition.

Various combinations of DC-chol A, DC-chol B and DOPE are useful in the practice of this invention, with amounts expressed in mole per cent of total lipid. TCB preparations are best comprised of from about 20 to about 80 mol% DOPE, and particularly from about 30 to 50%, and more particularly about 40%. In particular embodiments about 50 to 70 mol% are employed. DC-chol is most useful at from about 20 to about 80 mol%, and particularly from about 50 to 70%, as well as about 30 to 50 mol%, and more particularly about 60%.

Contemplated relative amounts of DC-chol A and B comprise a full spectrum from 100% A to 100% B. For example, halogenated solvent-free 100% DC-chol A preliposomal lyophilate can be prepared in 100% tertiary butyl alcohol (tBA). In particular embodiments, TCBs were prepared with about 40 mol% DOPE and about 60 mol% DC-chol. The DC-chol was employed at five principal molar ratios presented as DOPE:DC-chol A:DC-chol B. These were (a) 4\6\0; (b) 4\4.5\1.5; (c) 4\3\3; (d) 4\1.5\4.5; and (e) 4\0\6.

In the disclosure set forth, relative weights of lipid are generally in mol% of total lipid unless otherwise noted. Amounts of sugar-class compounds are generally expressed as weight per volume unless otherwise stated. Amounts of tBA are generally expressed in volume per /volume unless otherwise stated.

Formulations of TCBs with the foregoing ratios of DC-Chol A or B and DOPE were dissolved in a solvent mixture ranging from 100% tertiary-butyl alcohol to 30% tBA:70% water. Note that tBA is a non-halogenated solvent whose freezing point and vapor pressure are well suited for use in the practice of lyophilization. The formulated solutions of lipid and solvent were clear and essentially particle free. Such formulations in which the components form a clear solution lead to several advantages. Specifically, the solution can be sterile filtered and subsequently aseptically filled to give a sterile lyophilized vial of pre-liposomal TCB. This is a distinct advantage over the method disclosed by U.S. Pat. 5,283,185 (Epand et al.) which requires size reduction of the DC-Chol liposomes by mechanical stress prior to filtration. Additionally, in preparations of the instant invention in which the lipids are not in the form of liposomes prior to lyophilization, and which result in a non-liposomal lyophilate, allow the preparation of the lyophilized TCB without cryoprotectants, since no liposomal particles are present to cryoprotect during lyophilization.

In the practice of this invention, using sugars and sugar alcohols as bulking agents was advantageous. Specifically, the use of a low concentration of mannitol (about 1% to about 2% by weight) in the formulation provided a means of generating TCBs of smaller size compared to lyophilization without mannitol. The use of mannitol in this manner had several advantages: by adding bulk, it enhanced the rehydration of the TCB; and contributed to the osmolarity of the reconstituted TCB; in addition to reducing the rehydrated TCB particle size. In the practice of this invention, smaller cationic liposomes are preferred, however, our experience shows that TCBs in the range of about 300-1000nm are equivalent with respect to *in vitro* transfection. Another embodiment utilized sucrose at a concentration in the TCB preparation such that, when reconstituted to the desired volume with water, the TCB was iso-osmotic,

and the final sucrose concentration was 300mM. TCBs prepared in this manner rehydrated to relatively smaller sizes. Use of 30mM and 50mM sucrose in this manner allows for simplified reconstitution.

A primary application of TCB lies in the ability to deliver DNA* to cells. The mixing of the DNA* with the rehydrated TCB has been throughly investigated. One method is described below. Several factors affect the size stability of the TCB/DNA* complex including: their relative concentrations, the volume in which they were mixed, and the composition of the injectable solution. An example of a preferred embodiment is the following:

Preparation of a TCB/DNA* complex to give a final concentration of 25ug/mL DNA: 250nmoles/mL (155ug/mL) lipid where the DNA solution and the TCB solution were mixed in equal volume.

TCB dosage form was composed of a 30cc flint glass vial (a first vial) containing a total mg lipid equivalent 6.2 mg/vial with DC-chol B and DOPE in a 6:4 mole ratio, where the TCB was lyophilized with 1% mannitol, and then rehydrated with 20.0 mLs of 5.0% dextrose in water. This resulted in a final concentration of lipid in the vial equivalent to 500nmoles/mL (310ug/mL).

The DNA* was presented in a 30mL glass vial (the second vial) filled with 1mg of DNA in 1mL of Tris 3mM, 0.3mM EDTA in sterile water, resulting in a concentration of DNA* equivalent to 1000 ug/mL. To this second vial 19 mL of 5% dextrose in water was added to yield a concentration of the DNA equivalent to 50ug/mL.

Equal volumes of the DNA* vial contents and the TCB vial contents were gently mixed in a vial (the third vial) to give a final concentration of 25ug/mL DNA: 250nmoles/mL (155ug/mL) lipid.

The compositions of this invention possess valuable pharmacological properties. TCBs facilitate administration of DNA* as therapeutic agents or diagnostic agents. In some embodiments, DNA* so administered produces proteins after entry into the subject cells. Such proteins, in particular protocols are markers. Such compositions can display antineoplastic effects, antiviral effects, immunomodulation, hormone mediated effects or modulation of and

compensation for metabolic anomalies or defects. In human and veterinary medicine, these effects can be demonstrated, for example, using the method of directly introducing TCB's in association with DNA* into a subject. Useful routes of administration include intraperitoneal delivery, intrapleural, subdural, intrathecal, intramuscular, intratumor, subcutaneous, buccal, sublingual, intravenous ("i.v.") (which may be general or in a delivery loop to a more specific site), intraarterial, or parenteral delivery, and further including aerosols (e.g., nasal spray). In some embodiments i.v. delivery augments delivery to the reticuloendothelial system. In another embodiment, treatment is of cells by *in vitro* or *extracorporeal* methodology. In some applications based on *in vitro* or *extracorporeal* methodology, cells are treated outside of an animal and then reintroduced into the animal.

In particular protocols, such as certain protocols those treating highly proliferative cells, modulation of metabolic defects, or when provoking an immune response, treatment will not be (in most instances) dose dependent, beyond mere threshold considerations. Some uses (such as diagnostic uses with radiolabeled nucleotides), however, are more usually dose dependent.

The size of the TCB's is noteworthy. A characteristic of TCBs hydrated from preliposomal lyophilates with at least about 25% DC-chol B provides TCBs with a mean diameter of 550 ± 225nm when reconstituted, when the TCB lyophilate was prepared from 50:50 tBA:water mixture.

These compositions can be used to deliver intracellular therapeutics to treat a variety of conditions including without limitation (i) metabolic and genetic diseases, (ii) infectious diseases including bacterial and viral conditions, (iii) parasitic diseases, (iv) neoplastic diseases, as well as (v) delivery of vaccines against infectious diseases, tumors, and parasites. Yet further, these compositions contemplate treatment of AIDS, and those medical conditions which responding to variation in protein production. However, the composition and method of this invention is particularly useful for the amelioration of neoplastic conditions including cancer and psoriasis. It is further useful for

treating conditions associates with inappropriate, abnormal cell proliferation, (whether over or under producing) and non-native cell proliferation.

The compositions of this invention are generally administered to animals, including but not limited to livestock (such as cattle and poultry), household pets such as cats, and dogs, and also to humans, etc.

The pharmacologically active compositions of this invention can be processed in accordance with conventional methods of Galenic pharmacy to produce medicinal agents for administration to subjects, e.g., mammals including humans.

The compositions of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for oral, parenteral, inhalation or topical application which do not deleteriously react with the active compositions. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, buffer solutions, protein solutions (such as albumen), carbohydrates such as sugars or sugar alcohols including dextrose, sucrose and mannitol. The pharmaceutical preparations can be sterilized and/or mixed with substances and the like which do not deleteriously react with the active compositions. Vials or ampules are convenient unit dosages, such as one containing the preliposomal lyophilate, and, optionally, DNA*, such as a plasmid. In one embodiment, the preliposomal lyophilate is in a sterile vial to which is added plasmid DNA*, water and sugar or sugar alcohol. A particular mixture comprises water for hydration which is 5% dextrose.

Sustained or directed release compositions are particularly contemplated.

The wide variety of effects for which the DNA* is introduced yields an equivalently large number of useful dosages. The practitioner skilled in the art will appreciate that administration is usefully "titrated" up to an efficacious dose. Thus, it is convenient to administer small doses until a particular response is detected, to arrive at a final dosage.

It will be appreciated that the actual preferred amounts of active compositions in a specific case will vary by subject as well as by the specific

compositions being utilized, the particular compositions formulated, the mode of application, and the particular sites and organism being treated. Dosages for a given host can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compositions and of a known agent, e.g., by means of an appropriate, conventional pharmacological protocol. The present invention is further illustrated by the following non-limiting examples.

Example 1 -- TCB Preparation

DC-chol A:DC-chol B:DOPE was prepared into TCBs of the following constituents (presented as molar ratios): (a) 6\0\4; (b) 4.5\1.5\4; (c) 3\3\4; (d) 1.5\4.5\4; and (e) 0\6\4. All formulations were prepared at above 30°C and at atmospheric pressure. Mixing was via stir bar at approximately 500 rpm. All solutions were stirred until clear and then aliquotted. Forty aliquots of equal weight (total ingredients) of each solution were placed (one each) into 30cc flint vials with a 20mm opening. Each vial contained about 10mL prior to lyophilization.

Table 1

A:B:DOPE (molar)	DCchol A (g)	DCchol B (g)	DOPE (g)	tBA (g)
(a) 6\0\4	0.240	0.0	0.238	7.65
(b) 4.5\1.5\4	0.180	0.064	0.238	7.65
(c) 3\3\4	0.120	0.129	0.238	7.65
(d) 1.5\4.5\4	0.060	0.193	0.238	7.65
(e) 0\6\4	0.0	0.258	0.238	7.65

Each of the solutions (a) through (e) was lyophilized (FTS Dura-Top MP model TDS-2C-MP [Stone Ridge, NY]), and then hydrated (injected with ambient temperature diluent into an ambient temperature lyophilization vial of formulation with 10mL ultrapure water. A cloudy suspension of TCBs resulted, which was

tested for size by PCS. Following mixing with DNA*, the resulting TCB/DNA* complex was tested by via transfection bioassay, particle sizing and direct observation.

Transfection Assay: The luciferase transfection assay provides a means of determining the capability of TCB preparations to deliver plasmid DNA. In this case, the plasmid DNA encodes the firefly luciferase reporter gene and can be detected in a rapid and sensitive assay where the luciferase protein reacts with its substrate, luciferin, causing a release of light. The light intensity is a measure of the luciferase protein and therefore a measure of the delivery of plasmid DNA to cells.

Hydration of TCB and addition of DNA*: Plasmid DNA dissolved in 3mM Tris, 0.3mM EDTA buffer pH 8.0 was either added directly to TCBs or was diluted first in an appropriate dilution medium (such as 5% dextrose or other injectable fluids) and then added to the resuspended TCBs. Following gentle mixing the TCB/plasmid DNA complex held at room temperature for 10 min to 4 hours.

The TCB/plasmid DNA complex was then diluted with tissue culture medium and then added to tissue culture cells, such as CHO (Chinese hamster ovary) cells. This TCB/plasmid DNA complex was allowed to incubate with the cells for 4 hours at 37°C. The medium containing the TCB/plasmid DNA complex was removed from the cells and then the cells were incubated for an additional 2 days at 37°C to allow for expression of the luciferase protein by the cells that have taken up the plasmid DNA. When transfection was successful a crude protein extract from the cells reacted with luciferin substrate producing light which was detected with a luminometer (Berthod Lumat, model LB9501, Wallac, Inc, Gaithersburg, M.D.)

Luminescence was measured as raw light units (RLU) and a calibration curve for protein allowed calculation of specific activity as luminescence /protein RLU/mg protein.

Particle sizing: Particle sizing as a determination of particle diameter was performed using photon correlation spectroscopy (PCS) according to

manufacturers specifications (Brookhaven Instruments PCS, model B1-9000AT, Brookhaven, NY) interfaced to a Compaq PC. An Argon 633 nm cylindrical laser was used as the light source. All measurements were performed at 90°, the angle of lowest contaminant scattering.

Sample work-up for TCB involved resuspension to 2000 nmol/mL in various solvents, with particular reference to: 300 mM dextrose, 300mM sucrose, 300 mM mannitol, 150mM saline, 5% dextrose injection, and sterile water. Approximately 600 to 1000 μL was placed into 4 mL test glass culture tubes. Tubes were subsequently wiped down with methanol using a low lint Kim-wipe™, checked for scratches, and inserted into a decalin bath.

All samples were measured using a special test-tube adapter. Samples resided in a glass vat bathed in decalin at about 25°C. Samples were adjusted for counts and baseline using a test run by adjusting the first and last delays (time sample detection). Proper sample collection times yielded a sigmoidal curve with a less than 1% baseline difference. After adjustments, samples were measured for 60 seconds. Afterwards, a cumulant analysis was performed for which quadratic size and polydispersity data as well as Kcounts and percent baseline (measured vs. calculated) were recorded. Cumulant data was printed out and tabulated. An example summary of average for TCBs reconstituted in water and diluted as shown is given in Table 2.

Table 2

Diluent	Diam, nm	Polydisp.	Kcnts/sec.	Base % diff.
Control-water	674.0	0.277	200.013	0.196
Sucrose(300mM)	471.1	0.277	290.248	-0.223
Saline(150mM)	532.2	0.295	313.477	-0.498
Dextrose(5%)	464.5	0.242	280.840	0.039

(TCB was reconstituted in water and subsequently diluted with the listed diluent.)

Appearance of the vial and lyophilate cake were considered as a determinant of product quality and a predictor of hydratability. All five cakes of the present example were off-white in color. Some material adhered to the sides of the vials.

Hydration of the lypohilate was performed at room temperature (25°C) with water.

Table 3 sets forth the results of the inspection of the product of (a) through (e).

Table 3

A:B:DOPE (mole ratio)	transfection RLUx 10^3/mg	TCB mean dia. nm	lyophilate cake observation	hydration at 25°C
(a) 6\0\4	~1885	~700	thin (~1cm), cratered on top, top and sides concave, fibrous. tightly interwoven strands, white with residue on vial walls	suspension with particles which settle
(b) 4.5\1.5\4	~3375	~659	~1cm cake, concave center ~0.5cm;fibrous; interwoven strands, white with residue on vial walls	immediate suspension hand mixing
(c) 3\3\4	~2206	~414	mound shape with concave center, ~1cm cake, slightly concave sides; loosely interwoven strands, white with residue on vial walls	immediate suspension, hand mixing
(d) 1.5\4.5\4	~1788	~311	annular or toroidal cake; ~0.75cm thick; concave center; interwoven strands, white with residue on vial walls	immediate suspension, hand mixing
(e) 0\6\4	~1032	~273	slight concave depression; highly fibrous with some tight and some loose interwoven strands, white with residue on vial walls	immediate suspension, hand mixing

Example 2 --DC chol A in 100% tBA

As described in Example 1, TCBs of DC-chol A and DOPE (without DC-chol B) were made in 100% tBA. This particular embodiment has been prepared in 30-cc, flint glass vials with an inner diameter of ~ 3.25 cm, in 30-cc vials with an inner diameter of ~ 2.50 cm and in 5-cc vials. The TCB lyophilates prepared with 100% DC-chol A showed some relationship to cake thickness with a thinner cake giving a preferred rehydrated TCB. The preparations made in the 30-cc, 3.25-cm ID vials and the 5-cc vials always a preferred TCB solution when hydrated. Those prepared in 2.50-cm ID vial, in some instances, generate particulates. Brief sonication of the solution, however, often reduces the degree of particulation. A typical example of embodiments made in 30-cc vials of ID ~3.25 cm is a TCB size of 723 ± 37 nm and a transfection result of 468 RLUx$10^3/\mu g$ when mixed with plasmid DNA. Samples (b) through (e) did not exhibit particulation upon hydration, regardless of vial type.

Example 3 -- DC-chol B in 100% to 30% tBA

Four different tBA/$H_2$0 systems were used to prepare vesicles: 10/0, 8/2, 5/5 and 3/7 (v/v). Formulations were made using 12 μmol of DC-chol B and 8 mmol of DOPE dissolved in 10 mL of each of the four solvents mixtures. Lyophilates were then prepared as in Example 1, but using a cycle designed to take the preliposomal solutions from 25°C down to -40°C and ramped over various time periods back to 25°C. One such cycle is presented in Fig. 3. In Fig 3, the solid line represents temperature and the dotted ;line represents pressure. Lyophilization was at atmospheric pressure (760000 mTorr) during freezing [graphed at 3000 m Torr for scale convenience), 300m Torr for primary drying, and then approximately 15mTorr or mm Hg of pressure was applied during the later stages (secondary drying -- from about 0°C to about 25°C) to achieve complete drying. Complete lyophilization was accomplished over about 3 to 4 days. All four solvent systems yielded a lyophilized product similar to those of Example 1, (b) through (e).

In applications using 100% tBA, caution must be employed because its freezing point is 23°C. Thus care in aliquotting into the lyophilization vials is indicated.

A particular embodiment with favorable characteristics of appearance and ease of hydration of the lyophilized cake in conjunction with UV turbidity data was a solvent system of a 50/50 (v/v) mix of tBA/$H_2$0 (see Example 7). Favorable appearance was that of a compact off-white cake formed with interwoven strands and a concave depression on the top. The cake and any material on the sides of the vial are readily rehydrated in the chosen aqueous solution with hand agitation or brief sonication to a cloudy suspension of TCBs without visible aggregation.

Example 4 -- Preparation of transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol

TCB was a 0\6\4 DC-chol A\DC chol B\DOPE lipid mixture. To produce a forty-vial batch of TCB, 1.2 μmol of DC-chol B and 0.8 μmol of DOPE powders were dispensed by mass into a flint glass vials. Into each vial was aliquotted 38.3 g of tBA. The resulting mixture was stirred at approx. 500 rpm on a heated (to ~30°C) plate until total dissolution of the lipids. After the solution became clear, 49.4 g of ultrapure water was added to the reaction bottle with mixing. Again the mixture was stirred to clarity to ensure homogeneity. This took about 20-30 min for volumes ≥ 500 mL. After thorough stirring, the

mixture was aliquotted by mass individually into the forty labeled vials, the vials were loosely stoppered, placed in the lyophilizer and then lyophilized to dryness, using the lyophilization program described in Example 3. The vials were then stoppered tightly under vacuum, removed from the lyophilizer and sealed.

Example 5: Use of Sugar-class compounds in TCB

Sugars have played a two-fold part in the developing formulation: that of a potential bulking agent (pre-lyophilization) and that of a diluent (post-lyophilization).

Example 5A -- Use of Sugar and Sugar Alcohols as Bulking Agents

Absent bulking agents, the lyophilates of this invention (lipidic cakes which result after lyophilization) were generally compact and flat. A flat cake, while useful, was less convenient for monitoring the temperature of the cake. Difficulty arose in maintaining intimate contact of the lyophilate with a thermocouple during lyophilization. Sugar-class compounds do not dissolve in 100% tBA and therefore do not integrate well into a totally non-aqueous formulation. Sugar crystals tend to remain as crystals throughout lyophilization. When using sugar-class compounds it was preferable to first dissolve the compound in water before combining it with the remainder of the formulation ingredients.

Sucrose, mannitol, dextrose and xylose were integrated into the formulation and then lyophilized, with varying cake results. When hydrated with ultrapure water, samples of 0\6\4 TCBs ranged in size (by PCS) on average from 223 nm to 542 nm. Table 4 below. Turbidity measurements (by the method of Example 7) showed that mannitol at a concentration of 300 mM, upon hydration, yielded less stable TCBs over time compared with dextrose or non-sugar-class compound TCBs. mannitol in lesser concentrations, 1% (55 mM)]better maintained TCB size stability.

Table 4

Sugar in Lyophilate {concentration = 300 mM}	PCS diameter, nm	Transfection Bioassay RLUx10³/μg
none (control)	516	276
mannitol	542	479, 880 *
sucrose	223	457
xylose	320	472
dextrose	353	358

* mean of two measurements

Studies of the post-lyophilization use of sugar-class compounds as diluent (hydrating) solutions were performed in tandem with the use of pre-lyophilization sugar-class compounds in formulation. In terms of mannitol-laden TCB, when made with mannitol to a final concentration 1% (55 mM), the resultant TCBs, when hydrated, behave well. The cakes hydrate with ease, the cakes were fluffy and full, with pharmacologically suitable TCB size stability. These formulations exhibited enhanced size stabilization when the TCB was mixed with the plasmid DNA. Mannitol-free TCBs (no sugar-class compounds) averaged 515 nm in diameter when formed and 884 nm when complexed with DNA*. TCBs with 1% mannitol averaged 407 nm in diameter when formed and 913 nm when complexed with DNA*.

Among TCB complexed plasmid sizes studied (via PCS), preferred TCB formulations included: 1% mannitol, 2% mannitol, and no sugar (control). Mannitol was selected as a preferred sugar for its properties as a bulking agent and ease of reconstitution and smaller relative liposomal average size (400 nm to 600 nm). As a complex, the control (no sugar in lyophilate) measured approximately 800 to 1100 nm on average. While the no sugar product was useful, the bulk contributed by the presence of sugars made manipulation of the lyophilate easier. No noticeable size differences have been observed in either 1% and 2% mannitol. Sizes consistently average between 500 and 900 nm. Both freshly reconstituted TCB's and TCB complexed with DNA* (here, luciferase or EIA plasmid) were sized.

5B: Sugar-class compounds as a Diluent

In order to determine the effect which sugar or salt containing solutions had on TCBs when used to hydrate, ten solutions were tested as alternative diluents:

5% dextrose in water, 2.5% dextrose in ½-strength lactated Ringer's solution, sodium lactate in water, a 50/50 volume mix of 5% dextrose and sodium lactate, lactated Ringer's solution, 5% dextrose solution with 0.2% NaCl, 5% dextrose solution with 0.45% NaCl, 5% dextrose in lactated Ringer's injection solution, 0.9% saline, and 300 mOsm dextrose in water and 300 mOsm sucrose in water. These tests showed a range of compatibility with the TCB to perform as desired. Results from testing with the UV turbidity screening assay(as discussed in Example 7), and the transfection assay (Table 5) suggest a advantages of the use of 5% dextrose in water for reconstitution of the lyophilized TCB and prior to mixing with DNA, because this results in a relatively stable size of the complex of the over time following DNA* complexation.

Table 5

Sugar Diluent Solution	Transfection Assay Results, RLUx10^3
5% dextrose in water	301 ± 70
2.5% dextrose in ½-strength lactated Ringer's solution	377 ± 183
sodium lactate in water	396 ± 313
50\50 volume mix of 5% dextrose and sodium lactate	46 ± 5
lactated Ringer's solution	70 ± 25
5% dextrose solution with 0.2% NaCl	71 *
5% dextrose solution with 0.45% NaCl	245 *
5% dextrose in lactated Ringer's injection solution	304 ± 301
0.9% saline	232 ± 110
300 mOsm dextrose in water	342 ± 88

* only one replicate measurement was made

Example 6 -- Complex Stability

An important aspect of this invention is the preparation of a stable TCB/DNA* complex; that is stable complexes relative to complex size. DC-chol A Liposome/DNA* prepared as described in U.S. Pat. 5,283,185 (Epand) increase in size as a function of time. Such instability can affect the pharmaceutical utility and reproducibility of response. The effect of several variables on the size stability of the TCB/DNA* complex are listed below.

Table 6

	Effect on Stability
Decreasing the total lipid & DNA concentration	Increased
Decreasing the DNA:Lipid total mass ratio	Increased
Use of 3mM Tris pH8, 0.3 mM EDTA	Increased
Use of Mannitol as a bulking agent for TCB	Increased
Reconstitution of TCB with 5% Dextrose in water or with sterile water for injection	Increased
Reconstitution of TCB with Salts or Media	Decreased

Several methods have been used to determine the trends including, mixing with visual monitoring of aggregation upon mixing, turbidity, transfection and sizing by PCS.

Example 7 -- Monitoring size stability of the TCB/DNA* complex.

Turbidity was determined via Varian Cary 3e UV-VIS Spectrophotometer. Within its DOS-based kinetic program, measuring changes in absorbance over time. A stable liposomal solution is at a constant initial absorbance. As DNA* is added, liposomes form complexes with plasmid DNA*. TCB's complexed with DNA* alter the turbidity, or cloudiness of the solution increasing the particle size. Hence, a change in light scattering, or absorption result. The tracing of time vs absorbance demonstrates changes in the stability of the complex over several time points beginning at time zero (before DNA* addition). The goal is to achieve a TCB/DNA* complex which is size stable over time.

To test stability, TCBs are prepared as described in Example 3. Thereafter, samples were measured approximately every 1 to 5 minutes at 600 nm., for a period of two to four hours. Samples contain 500 nmol/mL TCB and 50 mg/mL plasmid DNA. Hydration and proper dilutions are made with 5% dextrose. Final concentrations and volumes have not been completely resolved. For lab-scale usage, samples are prepared in approximately 1 mL aliquots. The lipid component was (here DOPE and DC-chol) prepared in 2 mL flint injection vials. Plasmid is prepared in 1 mL micro-centrifuge tubes. Afterwards, plasmid is combined with TCB in the flint vials and inserted into pre-set sample cuvettes in a special application 12 cell multi-cell holder. A reference (blank) cuvette with 5% dextrose is included in cell twelve. Thereafter, the program is immediately

started. During sample work-up (DNA injection) time is kept, and consequently, added to the assay cycle time during data work-up.

An example of the utility of the assay follows. Samples were measured every 30 seconds for 45 to 60 minutes. Sample work-up included 30 μL TCB (2000 nmol/mL), 6 μL plasmid (1 μg/μL), and 564 μL diluent (identical to reconstitution solvent), a plasmid DNA: TCB ratio of 1:20. TCB's were reconstituted in various diluents including 300 mM dextrose, 300 mM mannitol, 300 mM sucrose, 150 mM saline, 150mM sodium lactate, 5% dextrose, and a 50/50 mixture of 5% dextrose and sodium lactate. Size stability data over time is presented as Fig. 4, wherein 150mM sodium lactate is represented as line (a), 5% dextrose as line (c), and the 50/50 mixture of 5% dextrose and sodium lactate as line (b). Selection of reconstitution solvent was based on (a) minimal increase in absorbance within the first 10 minutes (complexation), and (b) minimal *change* absorbance over the remaining assay as parameters indicative of size stability. An example diluent screening assay is given below.

Example 8 -- Preparation of DC-chol B

To a solution of N,N-dimethylethylenediamine (30.5mL, 24.7g, 278.4mmol), distilled from potassium hydroxide under nitrogen in chloroform (61mL) solution of cholesteryl chloroformate (20.8434g, 46.45mmol) in chloroform (121mL) was dropwise added at -10°C with energetic stirring. Reaction mixture was stirred for an additional 10 minutes and water (30.5mL) was dropwise added. The mixture was then transferred to a separatory funnel and washed three times with water (3x100ml) and dried over sodium sulfate. Thin layer chromatography ($CHCl_3$/MeOH 65:35, fr 0.6) revealed complete conversion of substrate. After filtering off sodium sulfate, solvents were evaporated *in vacuo* and crystallizing oil was dried *in vacuo* (16h, 0.1mbar, 20-25°C), yielding 23.3g of material. This material was then crystallized from an ethanol/acetonitrile mixture, cyclohexane, and again from ethanol/acetonitrile to yield 18.6171 grams of fine, colorless crystals of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol.

To a stirred solution of 4.8563g of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol in ethyl acetate (50mL) 1M solution of hydrogen chloride in ethyl acetate (9.7mL, 9.7mM) was dropwise added at 20-25°C. Reaction mixture was stirred for an additional 10 minutes and was diluted with ethyl acetate (30mL). Precipitate was then filtered through a Buchner funnel and washed with several portions of ethyl acetate (6x25 mL) until neutral pH. Precipitate was dried *in vacuo* (16h, 0.1mbar, 20-25°C to yield 4.8463g 3β[N-

(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride. This scheme is also set forth in Fig 2.

What is claimed:

1. The acid salt of 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol ("DC-chol B").

2. The composition of Claim 1 wherein said acid salt is the hydrochloride (HCl) salt.

3. A transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising the lipid 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol.

4. The lyophilate of Claim 3 wherein the lyophilate is cationic.

5. The lyophilate of Claim 3 wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is an acid salt.

6. The lyophilate of Claim 5 wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol acid salt is the hydrogenchloride salt.

7. The lyophilate of Claim 3 further comprising a lipid selected from the group consisting of dioleoyl phosphatidylethanolamine ("DOPE") and dioleoyl phosphatidylcholine ("DOPC").

8. The lyophilate of Claim 7 wherein said selected lipid is DOPE.

9. The lyophilate of Claim 8 wherein said DOPE constitutes from about 20 mol% to about 80 mol% of the total lipid of the lyophilate as compared with the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol.

10. The lyophilate of Claim 9 wherein said DOPE constitutes from about 30 mol% to about 50 mol% of the total lipid of the lyophilate.

11. The lyophilate of Claim 8 wherein said DC-chol constitutes from about 50 mol% to about 70 mol% of the total lipid of the lyophilate.

12. The lyophilate of Claim 3 further comprising a sugar-class compound.

13. The lyophilate of Claim 12 further wherein the a sugar-class compound is selected from the group comprising dextrose, mannitol, sucrose, lactose, mannose, and xylose.

14. The lyophilate of Claim 13 wherein the sugar-class compound is mannitol.

15. The solution of Claim 13 wherein the sugar-class compound is sucrose.

16. A halogenated solvent-free transfecting transmembrane compatible body comprising a 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol.

17. The halogenated solvent-free transfecting transmembrane compatible body of Claim 16 further comprising a lipid selected from the group comprising DOPE and DOPC.

18. The halogenated solvent-free transfecting transmembrane compatible body of Claim 17 wherein the selected lipid is DOPE.

19. The transfecting transmembrane compatible body of Claim 16 further comprising DNA*.

20. The halogenated solvent-free transfecting transmembrane compatible body of Claim 16 wherein said 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is basic.

21. The halogenated solvent-free transfecting transmembrane compatible body of Claim 16 wherein said 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is an acid salt.

22. The transfecting transmembrane compatible body of Claim 21 further comprising DNA*.

23. The halogenated solvent-free transfecting transmembrane compatible body of Claim 21 wherein said 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol acid salt is the hydrogenchloride salt.

24. The transfecting transmembrane compatible body of Claim 23 further comprising DNA*.

25. The transfecting transmembrane compatible body of Claim 16 wherein said TCB is cationic.

26. A halogenated solvent-free solution comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol wherein substantially all 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is solvated.

27. The solution of Claim 26 further comprising tBA.

28. The solution of Claim 27 comprising water and tBA wherein the ratio of water to tBA (v/v) is from about 70:30 to about 0:100.

29. The solution of Claim 28 wherein the percentage of water to tBA (v/v) is at least about 70%.

30. The solution of Claim 26 wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is at least about 25mol% DC-chol B.

31. The solution of Claim 30 wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is at least about 40mol% DC-chol B.

32. The solution of Claim 31 wherein the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol is at least about 60mol% DC-chol B.

33. The solution of Claim 26 further comprising a dissolved sugar-class compound.

34. The solution of Claim 33 further wherein the sugar-class compound is selected from the group comprising dextrose, mannitol, sucrose, lactose, mannose, and xylose.

35. The solution of Claim 34 wherein the sugar-class compound is mannitol.

36. The solution of Claim 34 wherein the sugar-class compound is sucrose.

37. A halogenated solvent-free process for preparing a transmembrane compatible body hydratable non-liposomal lyophilate comprising the lipid 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol) comprising the steps of

(a) solvating DC-chol in a halogenated solvent-free solvent;

(b) simultaneously or sequentially with step (a) solvating the lipid DOPE in said halogenated solvent-free solvent;

(c) lyophilizing said solutions of (a) and (b).

38. The process of Claim 37 wherein said DC-chol is DC-chol A and B in a ratio of from about 0:100 to about 100:0. (mol/mol)

39. The process of Claim 38 wherein said ratio is from about 20:80 to about 40:60.

40. The process of Claim 37 wherein said DOPE is present from about 20 mol% to about 80 mol% relative to the 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol as measured to total lipid in the lyophilate.

41. The process of Claim 40 wherein said DOPE is present from about 30 mol% to about 50 mol%.

42. The process of Clam 37 further comprising simultaneously or sequentially with steps (a) and (b) dissolving a sugar-class compound therein.

43. The process of Clam 42 wherein said sugar-class compound is selected from the group comprising dextrose, mannitol, sucrose, lactose, mannose, and xylose.

44. The process of Claim 43 wherein said sugar-class compound is mannitol.

45. The process of Claim 43 wherein said sugar-class compound is sucrose.

46. The process of Claim 44 wherein said mannitol is present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5% (w/v).

47. The process of Claim 46 wherein said mannitol is present in the solution prior to lyophilization in a ratio of from about 0.5% to about 2%.

48. The process of Claim 43 wherein said sugar-class compound is present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5% (w/v).

49. The process of Claim 48 wherein said sugar-class compound is present in the solution prior to lyophilization in a ratio of from about 0.5% to about 2%.

50. A method of incorporating a sugar-class compound into a transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol comprising

(a) solvating DC-chol in an halogenated solvent-free solvent;

(b) simultaneously or sequentially with step (a) solvating DOPE in said halogenated solvent-free solvent;

(c) simultaneously or sequentially with steps (a) and (b) dissolving a sugar-class compound in said halogenated solvent-free solvent;

(d) lyophilizing said solutions of (a)-(c).

51. The method of Clam 50 wherein said sugar-class compound is selected from the group comprising dextrose, mannitol, sucrose, lactose, mannose, and xylose.

52. The method of Claim 51 wherein said sugar-class compound is mannitol.

53. The method of Claim 51 wherein said sugar-class compound is sucrose.

54. The method of Claim 52 wherein said mannitol is present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5% (w/v).

55. The method of Claim 54 wherein said mannitol is present in the solution prior to lyophilization in a ratio of from about 0.5% to about 2%.

56. The method of Claim 51 wherein said sugar-class compound is present in the solution prior to lyophilization in a ratio of from about 0.1% to about 7.5% (w/v).

57. The method of Claim 56 wherein said sugar-class compound is present in the solution prior to lyophilization in a ratio of from about 0.5% to about 2%.

58. A method of introducing bioactive DNA* into a cell comprising the step of exposing said cell to a halogenated solvent-free TCB/DNA*complex wherein said TCB comprises 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol and DOPE, and is absent halogenated solvent.

59. A transmembrane compatible body hydratable non-liposomal halogenated solvent-free lyophilate comprising a lipid which is a cationic lipid acid salt.

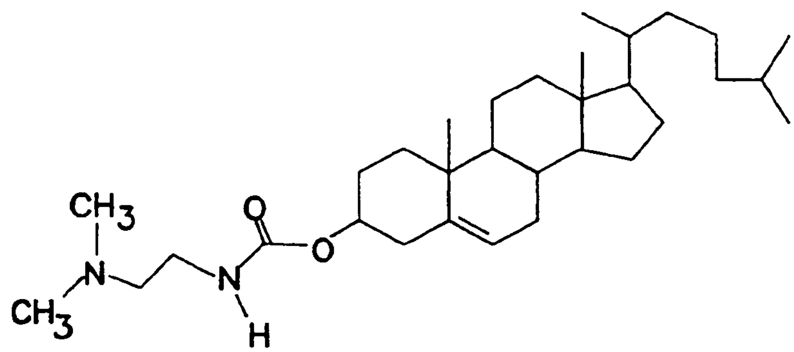
60. The lyophilate of Claim 59 further comprising a lipid selected from the group consisting of DOPE and DOPC.

61. The lyophilate of Claim 62 wherein the selected lipid is DOPE.

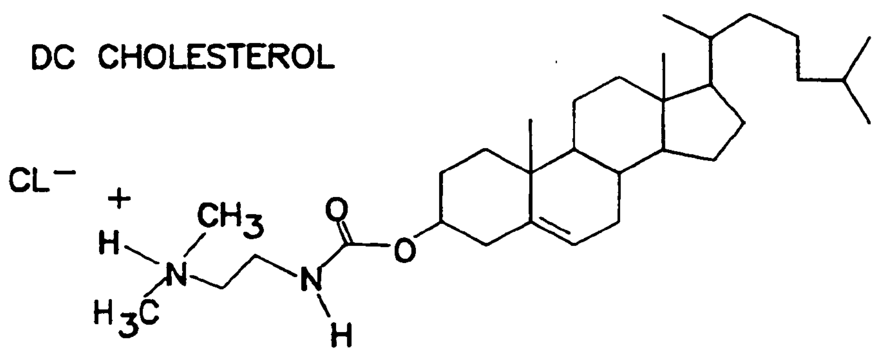
62. The lyophilate of Claim 61 wherein said DOPE constitutes from about 20 mol% to about 80 mol% of the lyophilate as compared with total lipid.

63. The lyophilate of Claim 59 further comprising a sugar-class compound.

64. The lyophilate of Claim 63 further wherein the sugar-class compound is selected from the group comprising dextrose, mannitol, sucrose, lactose, mannose, and xylose.



DC CHOLESTEROL



DC CHOLESTEROL HYDROCHLORIDE

FIG. 1

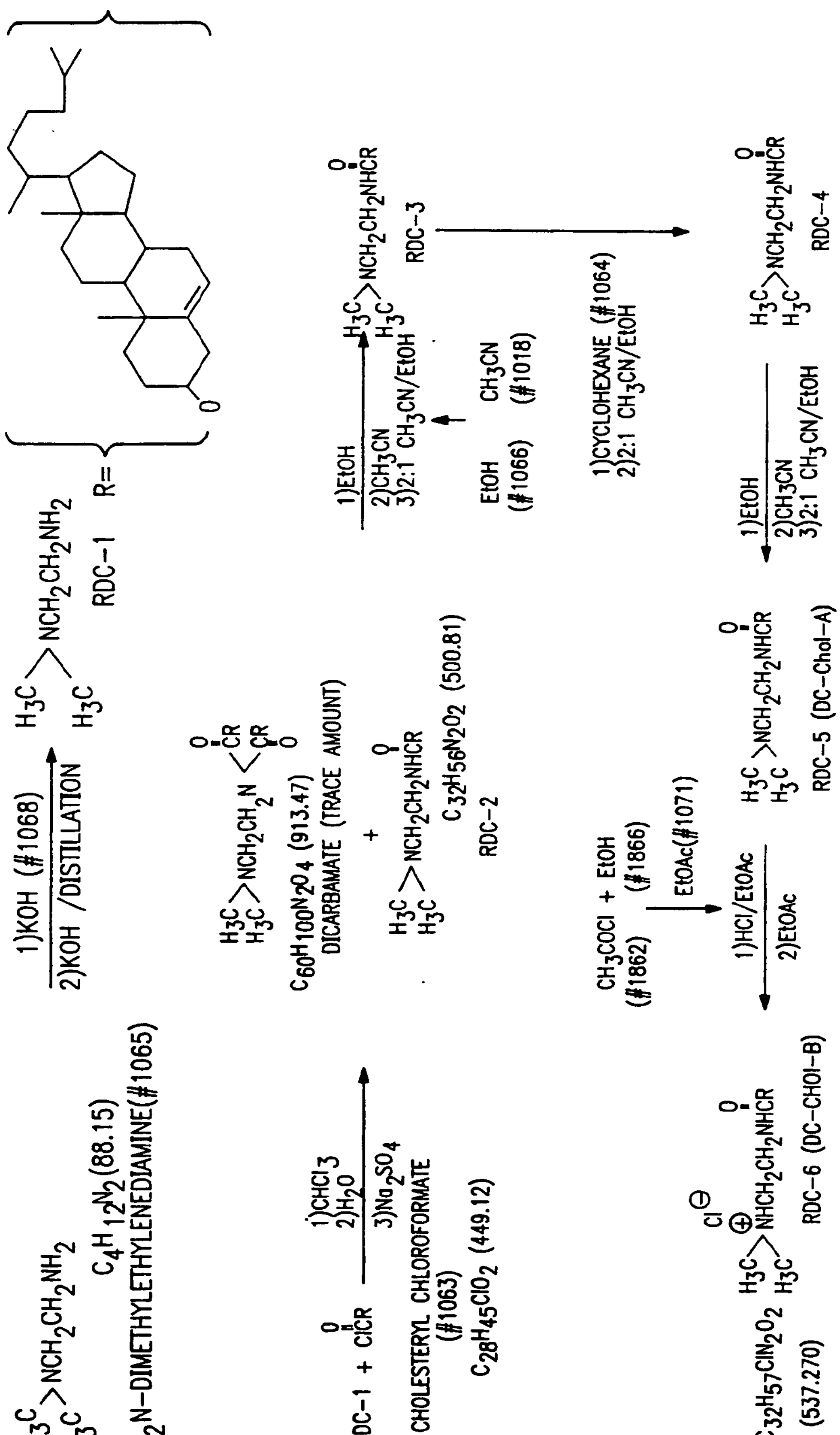

N,N-DIMETHYLETHYLENEDIAMINE(#1065)
C4H12N2 (88.15)
1)KOH (#1068)
2)KOH /DISTILLATION
RDC-1
R=
RDC-1 + ClCR
CHOLESTERYL CHLOROFORMATE (#1063)
C28H45ClO2 (449.12)
1)CHCl3
2)H2O
3)Na2SO4
C60H100N2O4 (913.47)
DICARBAMATE (TRACE AMOUNT)
C32H56N2O2 (500.81)
RDC-2
1)EtOH
2)CH3CN
3)2:1 CH3CN/EtOH
EtOH (#1066)
CH3CN (#1018)
RDC-3
1)CYCLOHEXANE (#1064)
2)2:1 CH3CN/EtOH
RDC-4
CH3COCl + EtOH
(#1862) (#1866)
EtOAc(#1071)
1)HCl/EtOAc
2)EtOAc
RDC-5 (DC-Chol-A)
C32H57ClN2O2
(537.270)
RDC-6 (DC-CHOl-B)

FIG. 2

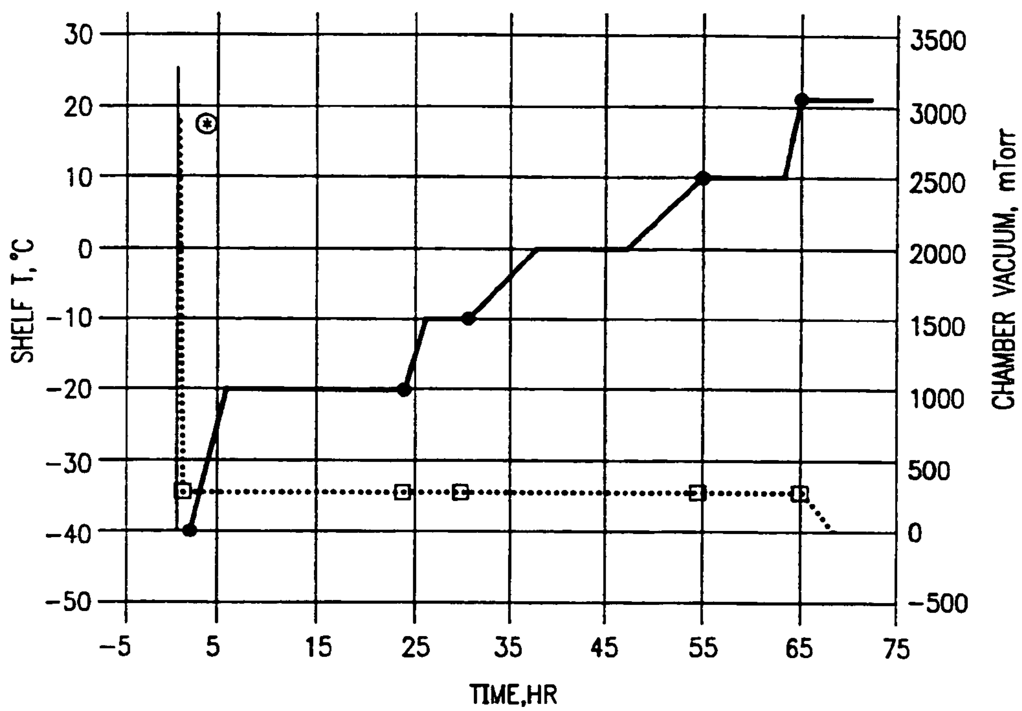

30
20
10
0
-10
-20
-30
-40
-50
SHELF T, °C
3500
3000
2500
2000
1500
1000
500
0
-500
CHAMBER VACUUM, mTorr
-5
5
15
25
35
45
55
65
75
TIME,HR

FIG. 3

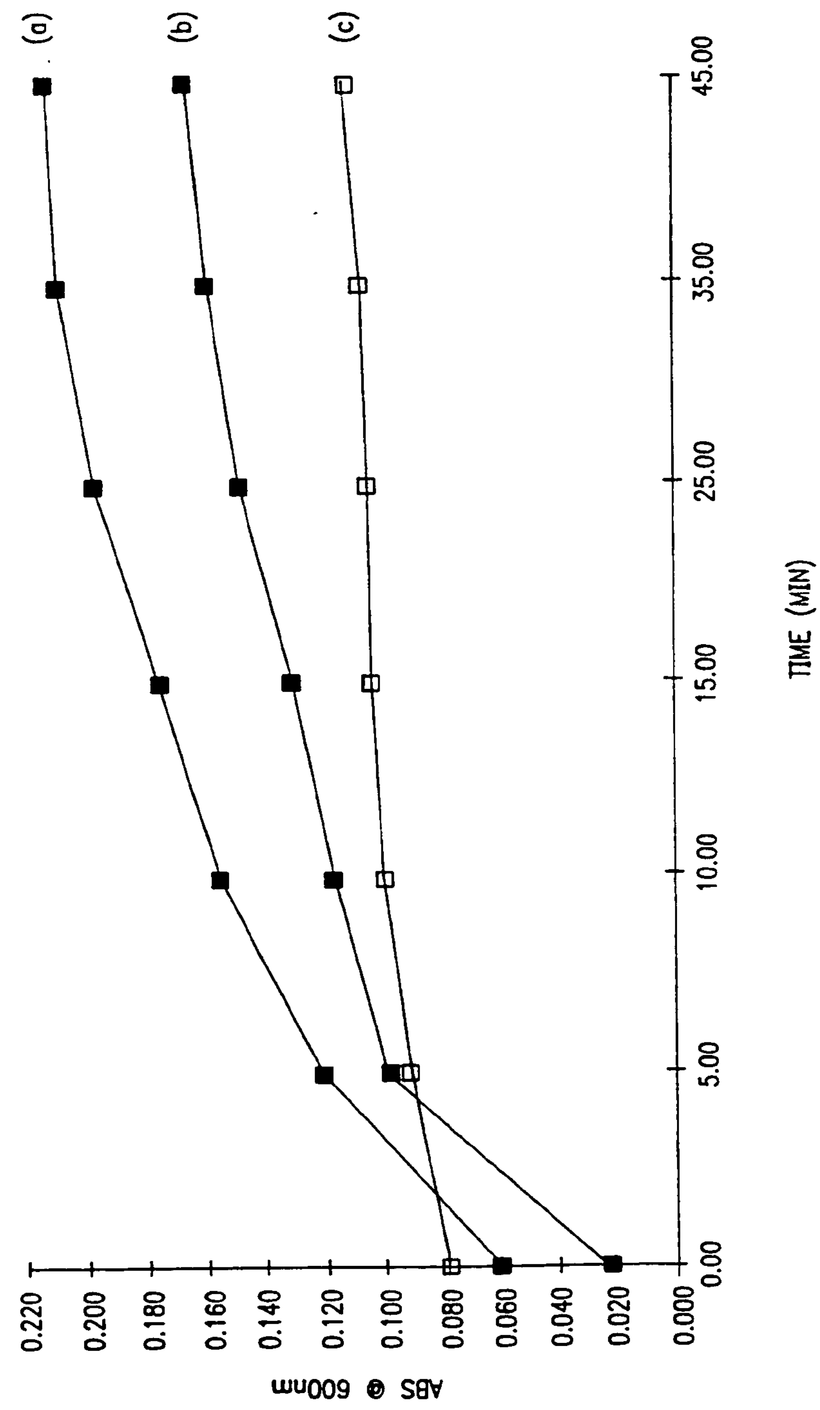

ABS @ 600nm
0.220
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0.00
5.00
10.00
15.00
25.00
35.00
45.00
TIME (MIN)
(a)
(b)
(c)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09954

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127;C12N 15/09
US CL : 424/450;935/54
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450;935/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
DC-CHOL, DIMETHYLAMINOETHANE CARBAMOYL CHOLESTEROL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,283,185 (EPAND ET AL.) 01 FEBRUARY 1994, COLUMNS 9-11 AND 14	3, 4, 7-20, 25, 59-65

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 AUGUST 1996

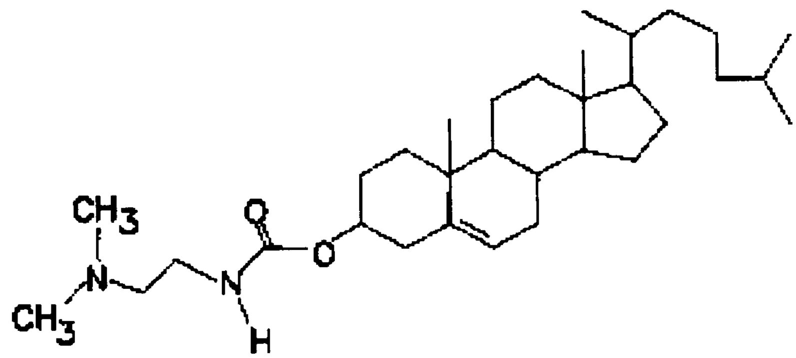
Date of mailing of the international search report

04 OCT 1996

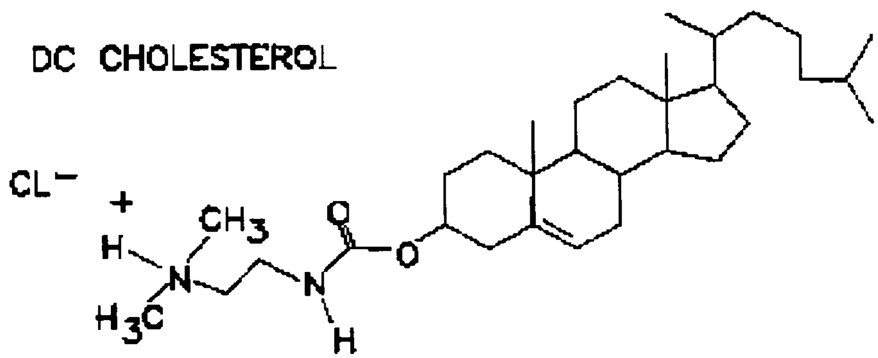
Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
JOHN S. BRUSCA
Telephone No. (703) 308-[illegible]

Form PCT/ISA/210 (second sheet)(July 1992)*



DC CHOLESTEROL



DC CHOLESTEROL HYDROCHLORIDE

FIG. 1

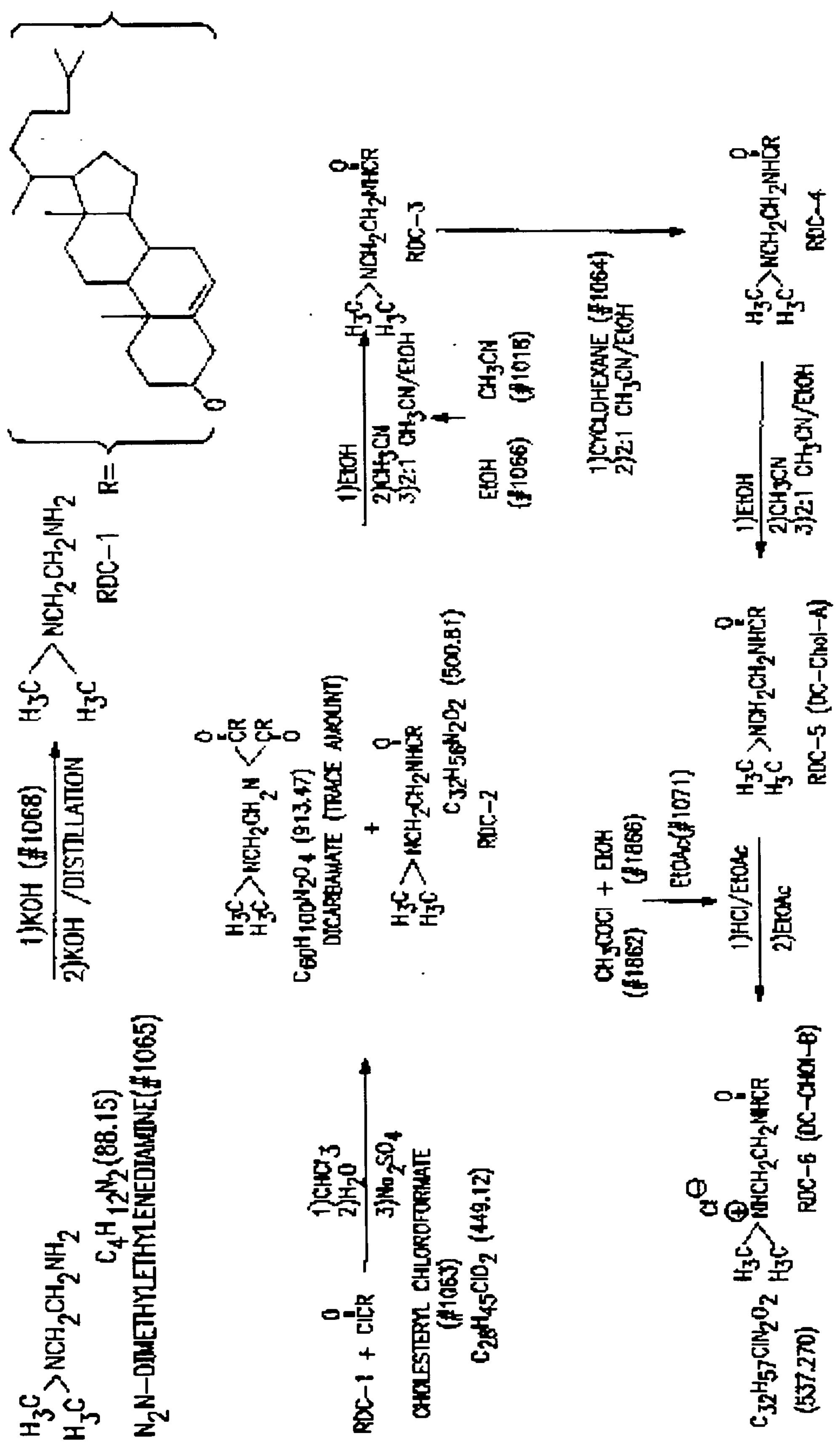

N,N-DIMETHYLETHYLENEDIAMINE(#1065)
1)KOH (#1068)
2)KOH /DISTILLATION
RDC-1
R=
CHOLESTERYL CHLOROFORMATE
(#1063)
1)CHCl3
2)H2O
3)Na2SO4
DICARBAMATE (TRACE AMOUNT)
RDC-2
RDC-3
RDC-4
RDC-5 (DC-Chol-A)
RDC-6 (DC-CHOl-B)
1)CYCLOHEXANE (#1064)
2)2:1 CH3CN/EtOH
1)HCl/EtOAc
2)EtOAc

FIG. 2

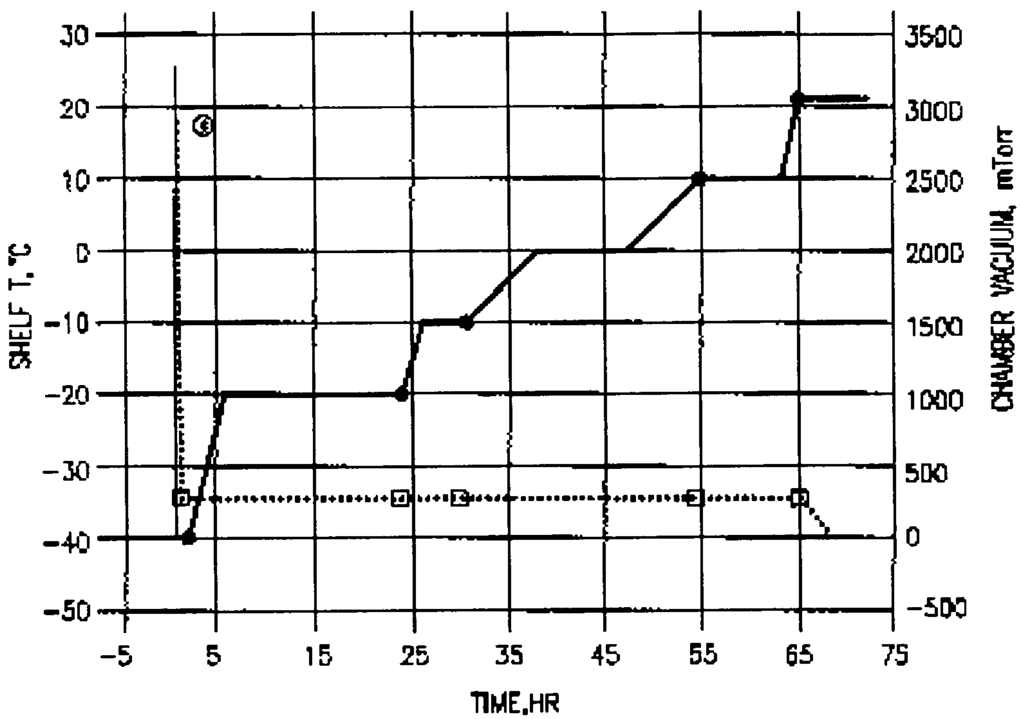

30
20
10
0
-10
-20
-30
-40
-50
SHELF T, °C
3500
3000
2500
2000
1500
1000
500
0
-500
CHAMBER VACUUM, mTorr
-5
5
15
25
35
45
55
65
75
TIME,HR

FIG. 3

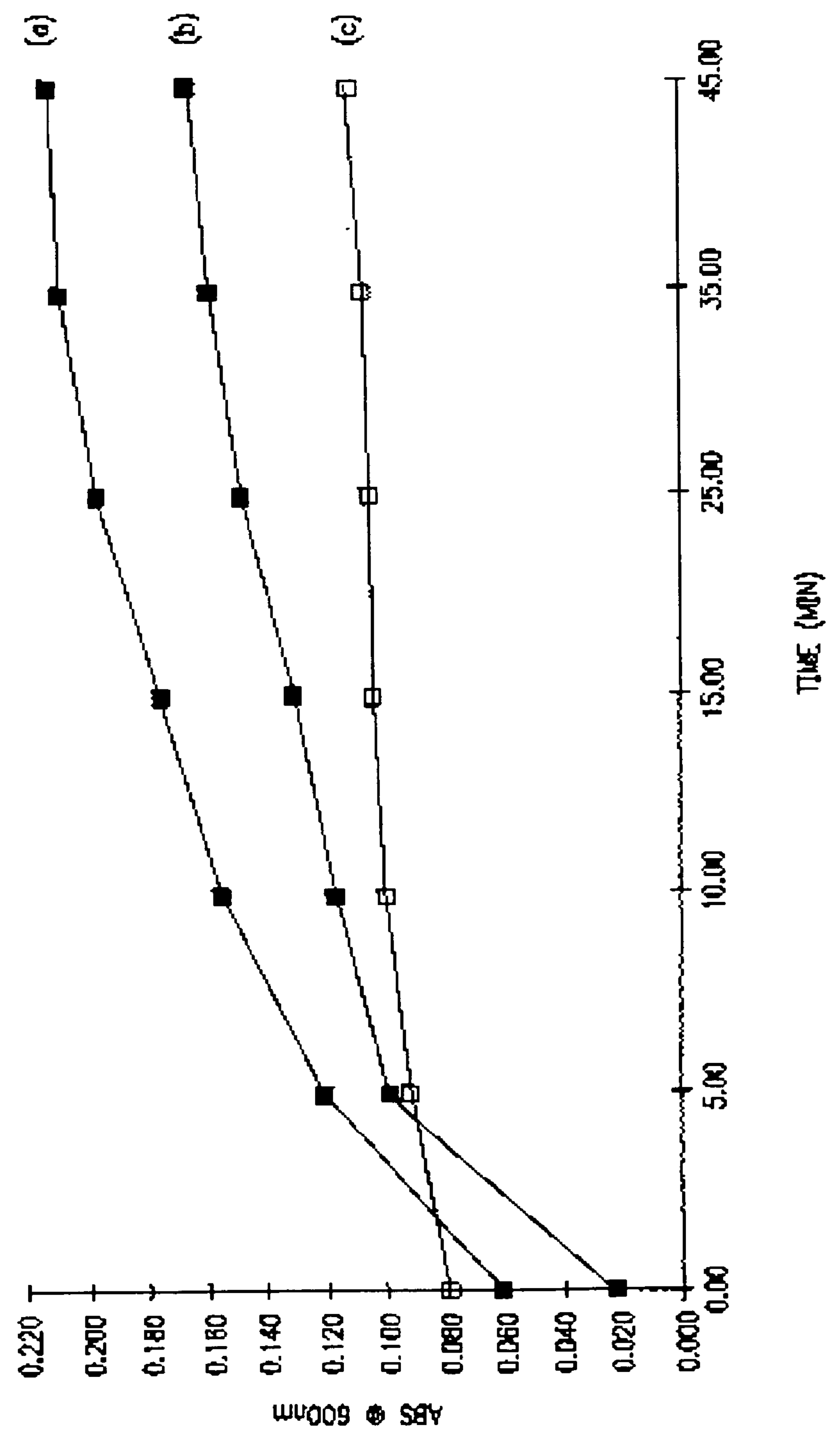

(a)
(b)
(c)
ABS @ 600nm
0.000
0.020
0.040
0.060
0.080
0.100
0.120
0.140
0.160
0.180
0.200
0.220
TIME (MIN)
0.00
5.00
10.00
15.00
25.00
35.00
45.00

FIG. 4